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Pam Mitchell

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PATENT

Pre Amendment A
P#3
2-12-02

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Philip E. Thorpe and Sophia Ran

Prior Serial No.: 09/351,543
Prior Filing Date: July 12, 1999

Serial No.: Unknown

Filing Date: November 30, 2001

For: COMBINED CANCER TREATMENT METHODS USING ANTIBODIES TO AMINOPHOSPHOLIPIDS

Group Art Unit: 1642

Examiner: Bansal, G.

Atty. Dkt. No.: 4001.002299

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination on the merits, the Examiner is respectfully requested to enter the following amendments. Remarks supporting patentability of all claims are also included, which the Examiner is respectfully requested to consider. All claims are believed to be condition for allowance, and examination and consideration is respectfully requested on this basis.

AMENDMENT

In the Specification:

In the title, please delete the existing title and replace this with a replacement title that reads:

COMBINED CANCER TREATMENT METHODS USING ANTIBODIES TO AMINOPHOSPHOLIPIDS

At page 1, after the heading 'Background of the Invention', please delete the existing paragraph or section of the Application pertaining to "Cross-reference to Related Applications" and replace such deleted paragraph or section with the following replacement paragraph or section:

The present application is a continuation of co-pending U.S. patent application Serial No. 09/351,543, filed July 12, 1999, which claims priority to first provisional application Serial No. 60/092,672, filed July 13, 1998, and second provisional application Serial No. 60/110,608, filed December 02, 1998, the entire text and figures of which applications are incorporated herein by reference without disclaimer. The U.S. Government owns rights in the present invention pursuant to grant numbers 1RO1CA74951-01 and 5RO1CA54168-05 from the National Institutes of Health.

In the Claims:

Upon filing the present continuation, please cancel claims 1-3, 11-22, 28-40 and 42-48 without prejudice and without disclaimer.

Please amend claims 4-10, 23 and 25, so that the rewritten claims read as follows:

4. (Amended) A method for treating an animal having a vascularized tumor, comprising simultaneously or sequentially administering to said animal a therapeutically effective

combination of at least a first pharmaceutical composition comprising at least a first antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor and at least a second therapeutic agent; wherein said second therapeutic agent is:

(a) a chemotherapeutic agent selected from the chemotherapeutic agents listed in Table B;

(b) an anti-angiogenic agent selected from the anti-angiogenic agents listed in Table C;

(c) an inflammatory cytokine, H_2O_2 or thrombin;

(d) a compound that interferes with tubulin activity; or

(e) a calcium flux inducing agent.

5. (Amended) The method of claim 4, wherein said at least a first antibody, or antigen-binding fragment thereof, binds to phosphatidylethanolamine on the luminal surface of blood vessels of the vascularized tumor.

6. (Amended) The method of claim 4, wherein said at least a first antibody, or antigen-binding fragment thereof, binds to phosphatidylserine on the luminal surface of blood vessels of the vascularized tumor.

7. (Amended) The method of claim 4, wherein said at least a first antibody is an IgG or IgM anti-aminophospholipid antibody.

8. (Amended) The method of claim 4, wherein said at least a first antigen-binding fragment of an antibody is an scFv, Fv, Fab', Fab or $F(ab')_2$ antigen-binding fragment of an anti-aminophospholipid antibody.

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9. (Amended) The method of claim 4, wherein said at least a first antibody is a human, humanized or part-human chimeric anti-aminophospholipid antibody or antigen-binding fragment thereof.

10. (Amended) The method of claim 4, wherein said at least a first antibody is an anti-aminophospholipid monoclonal antibody or antigen-binding fragment thereof.

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23. (Amended) The method of claim 4, wherein said at least a first antibody is a dimer, trimer or multimer of an anti-aminophospholipid antibody or antigen-binding fragments thereof.

#4

25. (Amended) The method of claim 4, wherein said at least a first pharmaceutical composition is administered to said animal via intravenous administration.

Upon filing the present continuation, please add claims 49-68, as follows:

49. (New) The method of claim 4, wherein said at least a second therapeutic agent is a chemotherapeutic agent selected from the chemotherapeutic agents listed in Table B.

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50. (New) The method of claim 49, wherein said at least a second therapeutic agent is cytosine arabinoside, methotrexate, aminopterin, demecolcine, mithramycin, chlorambucil,

melphalan, daunorubicin, doxorubicin, verapamil, tamoxifen, taxol, vincristine, vinblastine, etoposide, 5-fluorouracil (5FU), camptothecin, actinomycin-D, mitomycin C, cisplatin, a combretastatin or cyclophosphamide.

51. (New) The method of claim 4, wherein said at least a second therapeutic agent is an anti-angiogenic agent selected from the anti-angiogenic agents listed in Table C.

52. (New) The method of claim 51, wherein said at least a second therapeutic agent is angiostatin or endostatin.

53. (New) The method of claim 4, wherein said at least a second therapeutic agent is an inflammatory cytokine.

54. (New) The method of claim 53, wherein said at least a second therapeutic agent is interleukin-4.

55. (New) The method of claim 4, wherein said at least a second therapeutic agent is H_2O_2.

56. (New) The method of claim 4, wherein said at least a second therapeutic agent is thrombin.

57. (New) The method of claim 4, wherein said at least a second therapeutic agent is a compound that interferes with tubulin activity.

58. (New) The method of claim 57, wherein said at least a second therapeutic agent is taxol, vincristine, vinblastine, bleomycin, or a combretastatin.

59. (New) The method of claim 4, wherein said at least a second therapeutic agent is a calcium-flux inducing agent.

60. (New) The method of claim 60, wherein said at least a second therapeutic agent is a calcium ionophore.

61. (New) The method of claim 4, wherein said at least a first antibody and said at least a second therapeutic agent are administered to said animal simultaneously.

62. (New) The method of claim 4, wherein said at least a first antibody and said at least a second therapeutic agent are administered to said animal sequentially.

63. (New) The method of claim 62, wherein said at least a second therapeutic agent is administered to said animal at a biologically effective time prior to said at least a first antibody.

64. (New) The method of claim 63, wherein said at least a second therapeutic agent injures or induces apoptosis in the endothelium of the blood vessels of said vascularized tumor.

65. (New) The method of claim 64, wherein said at least a second therapeutic agent is taxol, vincristine, vinblastine, neomycin, a combretastatin, a podophyllotoxin, TNF-α, angiostatin, endostatin, vasculostatin, an $\alpha_v\beta_3$ antagonist, a calcium ionophore or a calcium-flux inducing agent; or is a prodrug thereof.

66. (New) The method of claim 62, wherein said at least a second therapeutic agent is administered to said animal at a biologically effective time subsequent to said at least a first antibody.

67. (New) The method of claim 66, wherein said at least a second therapeutic agent is an anti-tumor cell immunotoxin or an anti-angiogenic agent.

68. (New) A method for treating cancer, comprising simultaneously or sequentially administering to an animal having a vascularized tumor a therapeutically effective combination of an unconjugated antibody that binds to an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor and at least a second therapeutic agent.

REMARKS

I. Continuing Application Status

The present application is a continuation of allowed, co-pending application Serial No. 09/351,543, filed July 12, 1999 ("the '543 application"; Attorney Docket No. 4001.002200). The inventorship remains the same as the earlier application.

The '543 application was filed with claims 1-48, directed to therapeutic methods that comprise administering to an animal an effective amount of at least a first naked (unconjugated)

antibody, or antigen-binding region thereof, that binds to an aminophospholipid on the luminal surface of tumor vascular endothelial cells. All claims were examined without entry of a restriction or species election requirement. The original and certain new claims were progressed to allowance, and a copy of the allowed claims is enclosed (**Exhibit D**). Within the claims allowed, certain claims recited the simultaneous or sequential administration of a second anti-cancer agent along with the anti-aminophospholipid naked antibody.

This continuation is directed to further combined treatment methods, which complement those allowed in the parent application by reciting certain particular therapeutic agents for joint administration. The present claims are largely based on claims 4, 28 and 47 as allowed in the '543 application, but revised to reflect certain exemplary species of therapeutic agent from the specification.

Applicants respectfully request that the preceding amendments to the specification and claims be entered prior to substantive examination of this application. All of the amendments and additionally presented claims are fully supported by the original parent, and earlier priority application, to which priority is still properly claimed.

II. Status of the Claims

On filing the present continuation, claims 1-3, 11-22, 28-40 and 42-48 from the parent application have been cancelled without prejudice or disclaimer. Claims 4-10, 23 and 25 have been amended, to reflect the invention of the continuation. Claims 49-68 have been added, which are unified with the amended claims and fully supported by the original specification. Claims 4-10, 23-27, 41 and 49-68 are therefore in the case.

III. Compliance with 37 C.F.R. § 1.121

Copies of the pending claims are attached hereto as **Exhibit A** and **Exhibit B**. In accordance with 37 C.F.R. § 1.121, the claims have been labeled as "(Amended") or "(New)", where appropriate. **Exhibit A** provides a clean copy of the pending claims, whereas **Exhibit B** shows the changes with brackets and underlining.

The title of the present application has been changed to better reflect the claims. The proper claim for priority has been timely introduced into the specification by amendment of the opening paragraph at page 1.

The amendments to the title, specification and claims comply with 37 C.F.R. § 1.121, as the required instructions, text in clean form, and separate copies marked to show the changes using brackets and underlining are all submitted (see **Exhibit C** for specification, and **Exhibit B** for claims).

IV. The Claims are Allowable

The claims allowed in the parent application include dependent (claim 28) and independent (claim 47) claims directed to the simultaneous or sequential administration of at least a second anti-cancer agent in combination with the claimed naked antibody (**Exhibit D**). The second agents recited in the claims are chemotherapeutic, radiotherapeutic, anti-angiogenic and apoptosis-inducing agents (claims 29 and 48) and anti-tumor immunoconjugates (claims 30-40 and 48). Although several examples of anti-tumor immunoconjugates are recited in claims 30-40, the claims do not specify any particular examples of chemotherapeutic, radiotherapeutic or anti-angiogenic agents.

The present continuation is directed to combination treatment methods reciting particular examples of second therapeutic agents, as supported by the original specification. The pending

claims are thus directed to species of combined treatment methods that were disclosed, but not claimed in the parent application.

Although not necessary to support the claims, Applicants also provide **Exhibit E** for the record, which is a manuscript describing certain studies relevant to the present invention, submitted for publication on behalf of the inventors. **Exhibit E** includes data showing that various factors and tumor-associated conditions known to be present in the tumor microenvironment are able to cause PS translocation in cultured endothelial cells. Hypoxia/reoxygenation, acidity, thrombin, and inflammatory cytokines, such as IL-1α, IL-1β, TNFα and IFN, are all shown to induce PS exposure without causing cytotoxicity. Hydrogen peroxide is also shown to be a strong inducer of PS.

From the data in **Exhibit E**, the inventors propose that conditions inside tumors generate reactive oxygen species that could induce PS exposure. It is explained that the agents or conditions shown to induce PS are associated with a rise of intracellular Ca^{2+} concentration, which could cause PS accumulation on the external side of the membrane by activating the scramblase enzyme and/or by inhibiting aminophospholipid translocase. Moreover, combined treatment with inflammatory cytokines and hypoxia-reoxygenation are shown to have greater than additive effects, suggesting that factors may interact to give amplified effects on PS-exposure on tumor endothelium *in vivo*.

Irrespective of the data in **Exhibit E**, and the mechanistic discussions therein, the allowance of all claims in the parent application, and particularly the allowance of claims directed to combination treatment methods in general, supports a finding of patentability for the present claims, which recite further examples of combination treatment described in the specification. Given that all requirements of patentability have been addressed in the parent

application, the present claims should also be free from rejection. Applicants therefore urge that the claims be immediately progressed to allowance.

Should a Terminal Disclaimer be deemed necessary to secure allowance of the claims, a telephone call to the Applicants' undersigned representative is solicited in order that the Disclaimer can be supplied without delay.

V. Additional Support for the Claims

As described above, the present claims are directed to combination treatment methods using particular species of therapeutic agents within the scope of the sub-generic methods allowed in the parent application. Support for combination treatment clearly exists in claims 28 and 47 of the parent application, as well as throughout the specification, particularly in the extensive section of the specification entitled "Combination Therapies" (Section J, at pages 118-146). Other than as described below, the direct relationship between the pending claims and those of the parent application is evidenced in **Exhibit B** and **Exhibit D**. Additional support for the present claims in the specification exists as follows.

In claim 4, the term "simultaneously or sequentially" administering a therapeutically effective "combination" has direct support in original claim 47. The "at least a second therapeutic agent" is supported by allowed claims 28 and 47, as supplemented by the specification at least at page 19, line 17, page 107, line 25 and at page 118, lines 18-30.

In definitions (a) and (b) of claim 4, the therapeutic agents are defined as a chemotherapeutic agent selected from Table B and an anti-angiogenic agent selected from Table C, as supported by Tables B and C, beginning at pages 124 and 128 of the specification, respectively.

In claim 4(c), the therapeutic agent is defined as an inflammatory cytokine, H_2O_2 or thrombin. Inflammatory cytokines are described in the specification at page 121, lines 16-23, as supplemented by page 40, line 18 and in Example XIII. The use of H_2O_2 and thrombin is detailed in Example XIII, at pages 173-176 in particular.

Claim 4(d) recites that the therapeutic agent is a compound that interferes with tubulin activity, as supported by the specification at least at page 122, lines 23-24.

In claim 4(e), the therapeutic agent is defined as a calcium flux inducing agent, as recited in the specification at least at page 33, line 11 and at page 120, line 7.

The revisions to claims 5-10, 23 and 25 are clerical and/or grammatical, and are supported by the claims themselves.

Various of the new dependent claims simply recite alternatives (a) through (e) of claim 4 separate from the other alternatives, as follows: claim 49, 4(a); claim 51, 4(b); claims 53, 55 and 56, each 4(c); claim 57, 4(d); and claim 59, 4(e). The intervening claims then recite particular examples of certain agents, as below.

Claim 50 recites certain examples of chemotherapeutic agents from Table B and Section J1, and as supplemented by those recited at page 33, lines 9-11, at page 122, lines 10-26, and in the text bridging pages 139 and 140.

Claim 52 specifies the anti-angiogenic agent as angiostatin or endostatin, as described in the specification at pages 128, 130 and 131.

Claim 54 exemplifies the inflammatory cytokine as interleukin-4, as listed in the specification at page 40, line 18 and at page 121, line 18.

Claim 58 recites exemplary therapeutic agents known to interfere with tubulin activity, as taxol, vincristine, vinblastine, bleomycin, or a combretastatin, as described at page 122, second and fourth paragraphs, and as supplemented by page 125, fourth line from bottom.

Claim 60 exemplifies the calcium-flux inducing agent with reference to a calcium ionophore, as recited in the specification at page 33, line 11.

Dependent claims 61 and 62 separately recite the simultaneous and sequential administration from claim 4 (see also, pages 119-120).

Within sequential administration, the "prior" administration of the second agent, as recited in claims 63, 64 and 65, is described by the specification at least at page 33, lines 5-12, page 119, lines 12-14 and at page 120, lines 5-9.

The "subsequent" administration of the second agent, as recited in claims 66 and 67, is described by the specification at least from page 32, line 27 to page 33, line 3, and at page 120, lines 9-11.

Finally, independent claim 68 is similar to claim 4, but recites only the use of the intact antibody, to the exclusion of the antigen-binding fragment thereof, as supported by allowed claims 54 and 61 in the parent application and by original claims 4, 47 and 7.

The foregoing support confirms that no new matter is included within any of the pending claims.

VI. Formalities

Formal drawings are enclosed herewith. The sequence requirements and Applicants' initial duty of disclosure are also met (see paragraphs in the Request for Continuation and enclosed Statement and courtesy copies of sequence listing and 1449s).

No fees should be due in addition to the enclosed filing fees. However, should any additional fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason, the Assistant Commissioner is authorized to deduct said fees from Williams, Morgan & Amerson, P.C. Deposit Account No. 50-0786/4001.002299.

VII. Conclusion

In conclusion, Applicants submit that, in light of the foregoing remarks, the present claims are in condition for allowance and an early indication to this effect is respectfully requested. Should Examiner Bansal have any questions or comments, a telephone call to the undersigned Applicants' representative is earnestly solicited.

Respectfully submitted,

Shelley P.M. Fussey
Reg. No. 39,458
Agent for Applicants

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Houston, Texas, 77040
(713) 934-4079

Date: November 30, 2001

EXHIBIT A
PENDING CLAIMS (4001.002299)

4. (Amended) A method for treating an animal having a vascularized tumor, comprising simultaneously or sequentially administering to said animal a therapeutically effective combination of at least a first pharmaceutical composition comprising at least a first antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor and at least a second therapeutic agent; wherein said second therapeutic agent is:

(a) a chemotherapeutic agent selected from the chemotherapeutic agents listed in Table B;

(b) an anti-angiogenic agent selected from the anti-angiogenic agents listed in Table C;

(c) an inflammatory cytokine, H_2O_2 or thrombin;

(d) a compound that interferes with tubulin activity; or

(e) a calcium flux inducing agent.

5. (Amended) The method of claim 4, wherein said at least a first antibody, or antigen-binding fragment thereof, binds to phosphatidylethanolamine on the luminal surface of blood vessels of the vascularized tumor.

6. (Amended) The method of claim 4, wherein said at least a first antibody, or antigen-binding fragment thereof, binds to phosphatidylserine on the luminal surface of blood vessels of the vascularized tumor.

7. (Amended) The method of claim 4, wherein said at least a first antibody is an IgG or IgM anti-aminophospholipid antibody.

8. (Amended) The method of claim 4, wherein said at least a first antigen-binding fragment of an antibody is an scFv, Fv, Fab', Fab or $F(ab')_2$ antigen-binding fragment of an anti-aminophospholipid antibody.

9. (Amended) The method of claim 4, wherein said at least a first antibody is a human, humanized or part-human chimeric anti-aminophospholipid antibody or antigen-binding fragment thereof.

10. (Amended) The method of claim 4, wherein said at least a first antibody is an anti-aminophospholipid monoclonal antibody or antigen-binding fragment thereof.

23. (Amended) The method of claim 4, wherein said at least a first antibody is a dimer, trimer or multimer of an anti-aminophospholipid antibody or antigen-binding fragments thereof.

24. The method of claim 4, wherein at least a second antibody that binds to an aminophospholipid, or an antigen-binding fragment thereof, is administered to said animal.

25. (Amended) The method of claim 4, wherein said at least a first pharmaceutical composition is administered to said animal via intravenous administration.

26. The method of claim 4, wherein an image of the vasculature of said vascularized tumor is first obtained by administering to said animal a diagnostically effective amount of a detectably-labeled antibody, or antigen-binding fragment thereof, that binds to and identifies an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor.

27. The method of claim 4, further comprising subjecting said animal to surgery or radiotherapy.

41. The method of claim 4, wherein said animal is a human patient.

49. (New) The method of claim 4, wherein said at least a second therapeutic agent is a chemotherapeutic agent selected from the chemotherapeutic agents listed in Table B.

50. (New) The method of claim 49, wherein said at least a second therapeutic agent is cytosine arabinoside, methotrexate, aminopterin, demecolcine, mithramycin, chlorambucil, melphalan, daunorubicin, doxorubicin, verapamil, tamoxifen, taxol, vincristine, vinblastine, etoposide, 5-fluorouracil (5FU), camptothecin, actinomycin-D, mitomycin C, cisplatin, a combretastatin or cyclophosphamide.

51. (New) The method of claim 4, wherein said at least a second therapeutic agent is an anti-angiogenic agent selected from the anti-angiogenic agents listed in Table C.

52. (New) The method of claim 51, wherein said at least a second therapeutic agent is angiostatin or endostatin.

53. (New) The method of claim 4, wherein said at least a second therapeutic agent is an inflammatory cytokine.

54. (New) The method of claim 53, wherein said at least a second therapeutic agent is interleukin-4.

55. (New) The method of claim 4, wherein said at least a second therapeutic agent is H_2O_2.

56. (New) The method of claim 4, wherein said at least a second therapeutic agent is thrombin.

57. (New) The method of claim 4, wherein said at least a second therapeutic agent is a compound that interferes with tubulin activity.

58. (New) The method of claim 57, wherein said at least a second therapeutic agent is taxol, vincristine, vinblastine, bleomycin, or a combretastatin.

59. (New) The method of claim 4, wherein said at least a second therapeutic agent is a calcium-flux inducing agent.

60. (New) The method of claim 4, wherein said at least a second therapeutic agent is a calcium ionophore.

61. (New) The method of claim 4, wherein said at least a first antibody and said at least a second therapeutic agent are administered to said animal simultaneously.

62. (New) The method of claim 4, wherein said at least a first antibody and said at least a second therapeutic agent are administered to said animal sequentially.

63. (New) The method of claim 62, wherein said at least a second therapeutic agent is administered to said animal at a biologically effective time prior to said at least a first antibody.

64. (New) The method of claim 63, wherein said at least a second therapeutic agent injures or induces apoptosis in the endothelium of the blood vessels of said vascularized tumor.

65. (New) The method of claim 64, wherein said at least a second therapeutic agent is taxol, vincristine, vinblastine, neomycin, a combretastatin, a podophyllotoxin, TNF-α, angiostatin, endostatin, vasculostatin, an $\alpha_v\beta_3$ antagonist, a calcium ionophore or a calcium-flux inducing agent; or is a prodrug thereof.

66. (New) The method of claim 62, wherein said at least a second therapeutic agent is administered to said animal at a biologically effective time subsequent to said at least a first antibody.

67. (New) The method of claim 66, wherein said at least a second therapeutic agent is an anti-tumor cell immunotoxin or an anti-angiogenic agent.

68. (New) A method for treating cancer, comprising simultaneously or sequentially administering to an animal having a vascularized tumor a therapeutically effective combination of an unconjugated antibody that binds to an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor and at least a second therapeutic agent.

EXHIBIT B
PENDING CLAIMS (4001.002299)

Cancel claims 1-3

4. (Amended) A method for treating an animal having a vascularized tumor, comprising simultaneously or sequentially administering to said animal a therapeutically effective [amount] combination of at least a first pharmaceutical composition comprising at least a first antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor and at least a second therapeutic agent; wherein said second therapeutic agent is:

(a) a chemotherapeutic agent selected from the chemotherapeutic agents listed in Table B;

(b) an anti-angiogenic agent selected from the anti-angiogenic agents listed in Table C;

(c) an inflammatory cytokine, H_2O_2 or thrombin;

(d) a compound that interferes with tubulin activity; or

(e) a calcium flux inducing agent.

5. (Amended) The method of claim 4, wherein said [pharmaceutical composition comprises] at least a first antibody, or antigen-binding fragment thereof, [that] binds to phosphatidylethanolamine on the luminal surface of blood vessels of the vascularized tumor.

6. (Amended) The method of claim 4, wherein said [pharmaceutical composition comprises] at least a first antibody, or antigen-binding fragment thereof, [that] binds to phosphatidylserine on the luminal surface of blood vessels of the vascularized tumor.

7. (Amended) The method of claim 4, wherein said [pharmaceutical composition comprises] at least a first antibody is an IgG or IgM anti-aminophospholipid antibody.

8. (Amended) The method of claim 4, wherein said [pharmaceutical composition comprises] at least a first antigen-binding fragment of an antibody is an scFv, Fv, Fab', Fab or $F(ab')_2$ antigen-binding fragment of an anti-aminophospholipid antibody.

9. (Amended) The method of claim 4, wherein said [pharmaceutical composition comprises] at least a first antibody is a human, humanized or part-human chimeric anti-aminophospholipid antibody or antigen-binding fragment thereof.

10. (Amended) The method of claim 4, wherein said [pharmaceutical composition comprises] at least a first antibody is an anti-aminophospholipid monoclonal antibody or antigen-binding fragment thereof.

Cancel claims 11-22

23. (Amended) The method of claim 4, wherein said [pharmaceutical composition comprises] at least a first antibody is a dimer, trimer or multimer of an anti-aminophospholipid antibody or antigen-binding fragments thereof.

24. The method of claim 4, wherein at least a second antibody that binds to an aminophospholipid, or an antigen-binding fragment thereof, is administered to said animal.

25. (Amended) The method of claim 4, wherein said at least a first pharmaceutical composition is administered to said animal via intravenous administration.

26. The method of claim 4, wherein an image of the vasculature of said vascularized tumor is first obtained by administering to said animal a diagnostically effective amount of a detectably-labeled antibody, or antigen-binding fragment thereof, that binds to and identifies an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor.

27. The method of claim 4, further comprising subjecting said animal to surgery or radiotherapy.

Cancel claims 28-40

41. The method of claim 4, wherein said animal is a human patient.

Cancel claims 42-48

49. (New) The method of claim 4, wherein said at least a second therapeutic agent is a chemotherapeutic agent selected from the chemotherapeutic agents listed in Table B.

50. (New) The method of claim 49, wherein said at least a second therapeutic agent is cytosine arabinoside, methotrexate, aminopterin, demecolcine, mithramycin, chlorambucil, melphalan, daunorubicin, doxorubicin, verapamil, tamoxifen, taxol, vincristine, vinblastine, etoposide, 5-fluorouracil (5FU), camptothecin, actinomycin-D, mitomycin C, cisplatin, a combretastatin or cyclophosphamide.

51. (New) The method of claim 4, wherein said at least a second therapeutic agent is an anti-angiogenic agent selected from the anti-angiogenic agents listed in Table C.

52. (New) The method of claim 51, wherein said at least a second therapeutic agent is angiostatin or endostatin.

53. (New) The method of claim 4, wherein said at least a second therapeutic agent is an inflammatory cytokine.

54. (New) The method of claim 53, wherein said at least a second therapeutic agent is interleukin-4.

55. (New) The method of claim 4, wherein said at least a second therapeutic agent is H_2O_2.

56. (New) The method of claim 4, wherein said at least a second therapeutic agent is thrombin.

57. (New) The method of claim 4, wherein said at least a second therapeutic agent is a compound that interferes with tubulin activity.

58. (New) The method of claim 57, wherein said at least a second therapeutic agent is taxol, vincristine, vinblastine, bleomycin, or a combretastatin.

59. (New) The method of claim 4, wherein said at least a second therapeutic agent is a calcium-flux inducing agent.

60. (New) The method of claim 4, wherein said at least a second therapeutic agent is a calcium ionophore.

61. (New) The method of claim 4, wherein said at least a first antibody and said at least a second therapeutic agent are administered to said animal simultaneously.

62. (New) The method of claim 4, wherein said at least a first antibody and said at least a second therapeutic agent are administered to said animal sequentially.

63. (New) The method of claim 62, wherein said at least a second therapeutic agent is administered to said animal at a biologically effective time prior to said at least a first antibody.

64. (New) The method of claim 63, wherein said at least a second therapeutic agent injures or induces apoptosis in the endothelium of the blood vessels of said vascularized tumor.

65. (New) The method of claim 64, wherein said at least a second therapeutic agent is taxol, vincristine, vinblastine, neomycin, a combretastatin, a podophyllotoxin, TNF-α, angiostatin, endostatin, vasculostatin, an $\alpha_v\beta_3$ antagonist, a calcium ionophore or a calcium-flux inducing agent; or is a prodrug thereof.

66. (New) The method of claim 62, wherein said at least a second therapeutic agent is administered to said animal at a biologically effective time subsequent to said at least a first antibody.

67. (New) The method of claim 66, wherein said at least a second therapeutic agent is an anti-tumor cell immunotoxin or an anti-angiogenic agent.

68. (New) A method for treating cancer, comprising simultaneously or sequentially administering to an animal having a vascularized tumor a therapeutically effective combination of an unconjugated antibody that binds to an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor and at least a second therapeutic agent.

EXHIBIT C
REPLACEMENT SECTIONS

In the title, the addition is as shown:

COMBINED CANCER TREATMENT METHODS USING ANTIBODIES TO AMINOPHOSPHOLIPIDS

In the title, the final text is as follows:

COMBINED CANCER TREATMENT METHODS USING ANTIBODIES TO AMINOPHOSPHOLIPIDS

At page 1, after the heading 'Background of the Invention', in the paragraph or section of the Application pertaining to "Cross-reference to Related Applications", the deletions and additions are as shown:

The present application is a continuation of co-pending U.S. patent application Serial No. 09/351,543, filed July 12, 1999, which claims priority to first provisional application Serial No. 60/092,672, filed July 13, 1998, and second provisional application Serial No. 60/110,608, filed December 02, 1998, the entire text and figures of which applications are incorporated herein by reference without disclaimer. The U.S. Government owns rights in the present invention pursuant to grant numbers 1RO1CA74951-01 and 5RO1CA54168-05 from the National Institutes of Health.

At page 1, after the heading 'Background of the Invention', in the paragraph or section of the Application pertaining to "Cross-reference to Related Applications", the final text is as follows:

The present application is a continuation of co-pending U.S. patent application Serial No. 09/351,543, filed July 12, 1999, which claims priority to first provisional application Serial No. 60/092,672, filed July 13, 1998, and second provisional application Serial No. 60/110,608, filed December 02, 1998, the entire text and figures of which applications are incorporated herein by reference without disclaimer. The U.S. Government owns rights in the present invention pursuant to grant numbers 1RO1CA74951-01 and 5RO1CA54168-05 from the National Institutes of Health.

EXHIBIT D
ALLOWED CLAIMS
Serial No. 09/351,543 (4001.002200; UTSD:549)

1. (Amended) A method for killing tumor vascular endothelial cells, comprising administering to an animal having a vascularized tumor at least a first antibody, or antigen-binding region thereof, that binds to an aminophospholipid on the luminal surface of tumor vascular endothelial cells in an amount effective to kill said tumor vascular endothelial cells.

2. A method for inducing coagulation in tumor vasculature, comprising administering to an animal having a vascularized tumor a vessel-occluding amount of at least a first antibody, or antigen-binding region thereof, that binds to an aminophospholipid on the luminal surface of tumor vasculature.

3. (Amended) A method for treating an animal having a vascularized tumor, comprising administering to said animal a tumor-destructive amount of at least a first antibody, or antigen-binding region thereof, that binds to an aminophospholipid on the luminal surface of tumor vasculature.

4. A method for treating an animal having a vascularized tumor, comprising administering to said animal a therapeutically effective amount of at least a first pharmaceutical composition comprising at least a first antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor.

5. The method of claim 4, wherein said pharmaceutical composition comprises at least a first antibody, or antigen-binding fragment thereof, that binds to phosphatidylethanolamine on the luminal surface of blood vessels of the vascularized tumor.

6. The method of claim 4, wherein said pharmaceutical composition comprises at least a first antibody, or antigen-binding fragment thereof, that binds to phosphatidylserine on the luminal surface of blood vessels of the vascularized tumor.

7. The method of claim 4, wherein said pharmaceutical composition comprises at least a first IgG or IgM anti-aminophospholipid antibody.

8. The method of claim 4, wherein said pharmaceutical composition comprises at least a first scFv, Fv, Fab', Fab or $F(ab')_2$ antigen-binding fragment of an anti-aminophospholipid antibody.

9. The method of claim 4, wherein said pharmaceutical composition comprises at least a first human, humanized or part-human chimeric anti-aminophospholipid antibody or antigen-binding fragment thereof.

10. The method of claim 4, wherein said pharmaceutical composition comprises at least a first anti-aminophospholipid monoclonal antibody or antigen-binding fragment thereof.

11. The method of claim 10, wherein said pharmaceutical composition comprises at least a first anti-aminophospholipid monoclonal antibody, or antigen-binding fragment thereof, that is prepared by a preparative process comprising:

(a) preparing an anti-aminophospholipid antibody-producing cell; and

(b) obtaining an anti-aminophospholipid monoclonal antibody from said antibody-producing cell.

12. The method of claim 11, wherein said anti-aminophospholipid antibody-producing cell is obtained from a human patient having a disease associated with the production of anti-aminophospholipid antibodies.

13. The method of claim 11, wherein said anti-aminophospholipid antibody-producing cell is obtained by stimulating a mixed population of human peripheral blood lymphocytes with an immunogenically effective amount of an aminophospholipid sample.

14. The method of claim 11, wherein said anti-aminophospholipid antibody-producing cell is obtained by immunizing an animal with an immunogenically effective amount of an aminophospholipid sample.

15. The method of claim 14, wherein said anti-aminophospholipid antibody-producing cell is obtained by immunizing a transgenic mouse that comprises a human antibody library with an immunogenically effective amount of an aminophospholipid sample.

16. The method of claim 11, wherein said preparative process comprises:

(a) fusing said anti-aminophospholipid antibody-producing cell with an immortal cell to prepare a hybridoma that produces an anti-aminophospholipid monoclonal antibody; and

(b) obtaining an anti-aminophospholipid monoclonal antibody from said hybridoma.

17. The method of claim 11, wherein said preparative process comprises:

(a) immunizing an animal with an immunogenically effective amount of an aminophospholipid sample;

(b) preparing a collection of antibody-producing hybridomas from the immunized animal;

(c) selecting from the collection a hybridoma that produces an anti-aminophospholipid antibody; and

(d) culturing the selected hybridoma to provide the anti-aminophospholipid monoclonal antibody.

18. The method of claim 17, wherein an antigen-binding region of the anti-aminophospholipid monoclonal antibody is operatively attached to a human antibody framework or constant region.

19. The method of claim 17, wherein the immunized animal is a transgenic mouse that comprises a human antibody library and wherein the anti-aminophospholipid monoclonal antibody is a human monoclonal antibody.

20. The method of claim 11, wherein said preparative process comprises:

(a) obtaining anti-aminophospholipid antibody-encoding nucleic acids from said anti-aminophospholipid antibody-producing cell; and

(b) expressing said nucleic acids to obtain a recombinant anti-aminophospholipid monoclonal antibody.

21. The method of claim 11, wherein said preparative process comprises:

(a) immunizing an animal with an immunogenically effective amount of an aminophospholipid sample;

(b) preparing a combinatorial immunoglobulin phagemid library expressing RNA isolated from the spleen of the immunized animal;

(c) selecting from the phagemid library a clone that expresses an anti-aminophospholipid antibody; and

(d) expressing an anti-aminophospholipid antibody-encoding nucleic acid from said selected clone to provide a recombinant anti-aminophospholipid monoclonal antibody.

22. The method of claim 21, wherein the immunized animal is a transgenic mouse that comprises a human antibody library and wherein the recombinant anti-aminophospholipid monoclonal antibody is a recombinant human monoclonal antibody.

23. The method of claim 4, wherein said pharmaceutical composition comprises a dimer, trimer or multimer of an anti-aminophospholipid antibody or antigen-binding fragments thereof.

24. The method of claim 4, wherein at least a second antibody that binds to an aminophospholipid, or an antigen-binding fragment thereof, is administered to said animal.

25. The method of claim 4, wherein said pharmaceutical composition is administered to said animal via intravenous administration.

26. The method of claim 4, wherein an image of the vasculature of said vascularized tumor is first obtained by administering to said animal a diagnostically effective amount of a detectably-labeled antibody, or antigen-binding fragment thereof, that binds to and identifies an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor.

27. The method of claim 4, further comprising subjecting said animal to surgery or radiotherapy.

28. The method of claim 4, further comprising simultaneously or sequentially administering to said animal a therapeutically effective amount of at least a second anti-cancer agent.

29. The method of claim 28, wherein said at least a second anti-cancer agent is a chemotherapeutic, radiotherapeutic, anti-angiogenic or apoptosis-inducing agent.

30. The method of claim 28, wherein said at least a second anti-cancer agent is an antibody-therapeutic agent construct comprising a targeting antibody, or antigen-binding fragment thereof, that binds to a surface-expressed, surface-accessible or surface-localized component of a tumor cell, tumor stroma or tumor vasculature; said targeting antibody or fragment thereof operatively linked to a therapeutic agent.

31. The method of claim 30, wherein said targeting antibody, or antigen-binding fragment thereof, binds to a cell surface antigen of a tumor cell.

32. The method of claim 30, wherein said targeting antibody, or antigen-binding fragment thereof, binds to a component of tumor stroma.

33. The method of claim 30, wherein said targeting antibody, or antigen-binding fragment thereof, binds to a surface-expressed, surface-accessible, surface-localized, cytokine-inducible or coagulant-inducible component of intratumoral blood vessels of a vascularized tumor.

34. The method of claim 33, wherein said targeting antibody, or antigen-binding fragment thereof, binds to a surface-expressed component of intratumoral vasculature selected from the group consisting of an aminophospholipid, endoglin, a TGFβ receptor, E-selectin, P-selectin, VCAM-1, ICAM-1, PSMA, a VEGF/VPF receptor, an FGF receptor, a TIE, $\alpha_v\beta_3$ integrin, pleiotropin, endosialin and an MHC Class II protein.

35. The method of claim 33, wherein said targeting antibody, or antigen-binding fragment thereof, binds to a surface-localized component of intratumoral vasculature selected from the group consisting of VEGF/VPF, FGF, TGFβ, a ligand that binds to a TIE, a tumor-associated fibronectin isoform, scatter factor/hepatocyte growth factor (HGF), platelet factor 4 (PF4), PDGF and TIMP.

36. The method of claim 30, wherein said targeting antibody, or antigen-binding fragment thereof, is operatively linked to a cytotoxic agent.

37. The method of claim 36, wherein said targeting antibody, or antigen-binding fragment thereof, is operatively linked to a plant-, fungus- or bacteria-derived toxin.

38. The method of claim 37, wherein said targeting antibody, or antigen-binding fragment thereof, is operatively linked to deglycosylated ricin A chain.

39. The method of claim 30, wherein said targeting antibody, or antigen-binding fragment thereof, is operatively linked to a coagulation factor or to an antibody, or antigen-binding fragment thereof, that binds to a coagulation factor.

40. The method of claim 39, wherein said targeting antibody, or antigen-binding fragment thereof, is operatively linked to Tissue Factor, truncated Tissue Factor or a derivative thereof, or to an antibody, or antigen-binding fragment thereof, that binds to Tissue Factor, truncated Tissue Factor or a derivative thereof.

41. The method of claim 4, wherein said animal is a human patient.

42. A method for treating cancer, comprising administering to an animal having a vascularized tumor a therapeutically effective amount of at least a first pharmaceutical composition comprising at least a first naked antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid on the luminal surface of intratumoral blood vessels of the vascularized tumor.

43. (Amended) A method for treating cancer, comprising administering to an animal having a vascularized tumor at least a first pharmaceutical composition comprising an amount of at least a first unconjugated antibody effective to kill at least a portion of the tumor or tumor vasculature; wherein said first unconjugated antibody is an unconjugated antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid expressed on the luminal surface of tumor vascular endothelial cells.

44. A method for treating cancer, comprising administering to an animal having a vascularized tumor at least a first pharmaceutical composition comprising an amount of at least a first unconjugated antibody effective to occlude or destroy tumor blood vessels, as opposed to normal blood vessels; wherein said first unconjugated antibody is an unconjugated antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid expressed on the luminal surface of tumor vascular endothelial cells.

45. A method for treating cancer, comprising administering to an animal having a vascularized tumor at least a first pharmaceutical composition comprising an amount of at least a first unconjugated antibody effective to induce tumor necrosis; wherein said first unconjugated antibody is an unconjugated antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid expressed on the luminal surface of blood vessels of the vascularized tumor.

46. A method for treating cancer, comprising:

(a) forming an image of a vascularized tumor by administering to an animal having a vascularized tumor a diagnostically effective amount of a detectably-labeled antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor, thereby forming a detectable image of the tumor vasculature; and

(b) subsequently administering to said animal a therapeutically effective amount of at least a first antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid on the tumor blood vessel luminal surface and thereby destroys the tumor vasculature.

47. A method for treating cancer, comprising simultaneously or sequentially administering to an animal having a vascularized tumor a therapeutically effective combination of an unconjugated antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor and at least a second anti-cancer agent.

48. The method of claim 47, wherein said at least a second anti-cancer agent is a chemotherapeutic, radiotherapeutic, anti-angiogenic or apoptosis-inducing agent or an antibody-therapeutic agent construct comprising a therapeutic agent operatively attached to an antibody, or antigen-binding fragment thereof, that binds to a surface-expressed, surface-accessible, surface-localized, cytokine-inducible or coagulant-inducible component of tumor vasculature or tumor stroma.

49. A method for inducing cell death in tumor vascular endothelial cells, comprising administering to an animal having a vascularized tumor at least a first antibody, or antigen-binding region thereof, that binds to an aminophospholipid on the luminal surface of tumor vascular endothelial cells in an amount effective to induce cell death in tumor vascular endothelial cells.

50. A method for treating an animal having a vascularized tumor, comprising administering to said animal at least a first antibody, or antigen-binding region thereof, that binds to an aminophospholipid on the luminal surface of tumor blood vessels in an amount effective to destroy or occlude at least a portion of said tumor blood vessels upon binding to said aminophospholipid.

51. (New) A method for killing tumor vascular endothelial cells, comprising administering to an animal having a vascularized tumor at least a first antibody that binds to an aminophospholipid on the luminal surface of tumor vascular endothelial cells in an amount effective to kill said tumor vascular endothelial cells.

52. (New) A method for inducing coagulation in tumor vasculature, comprising administering to an animal having a vascularized tumor a vessel-occluding amount of at least a first antibody that binds to an aminophospholipid on the luminal surface of tumor vasculature.

53. (New) A method for treating an animal having a vascularized tumor, comprising administering to said animal a tumor-destructive amount of at least a first antibody that binds to an aminophospholipid on the luminal surface of tumor vasculature.

54. (New) A method for treating an animal having a vascularized tumor, comprising administering to said animal a therapeutically effective amount of at least a first pharmaceutical composition comprising at least a first antibody that binds to an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor.

55. (New) The method of claim 54, wherein said pharmaceutical composition comprises at least a first antibody that binds to phosphatidylserine on the luminal surface of blood vessels of the vascularized tumor.

56. (New) The method of claim 54, wherein said animal is a human patient.

57. (New) A method for treating cancer, comprising administering to an animal having a vascularized tumor a therapeutically effective amount of at least a first pharmaceutical composition comprising at least a first naked antibody that binds to an aminophospholipid on the luminal surface of intratumoral blood vessels of the vascularized tumor.

58. (New) A method for treating cancer, comprising administering to an animal having a vascularized tumor at least a first pharmaceutical composition comprising an amount of at least a first unconjugated antibody effective to kill at least a portion of the tumor or tumor vasculature; wherein said first unconjugated antibody binds to an aminophospholipid expressed on the luminal surface of tumor vascular endothelial cells.

59. (New) A method for treating cancer, comprising administering to an animal having a vascularized tumor at least a first pharmaceutical composition comprising an amount of at least a first unconjugated antibody effective to occlude or destroy tumor blood vessels, as opposed to normal blood vessels; wherein said first unconjugated antibody binds to an aminophospholipid expressed on the luminal surface of tumor vascular endothelial cells.

60. (New) A method for treating cancer, comprising administering to an animal having a vascularized tumor at least a first pharmaceutical composition comprising an amount of at least a first unconjugated antibody effective to induce tumor necrosis; wherein said first unconjugated antibody is an unconjugated antibody that binds to an aminophospholipid expressed on the luminal surface of blood vessels of the vascularized tumor.

61. (New) A method for treating cancer, comprising simultaneously or sequentially administering to an animal having a vascularized tumor a therapeutically effective combination of an unconjugated antibody that binds to an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor and at least a second anti-cancer agent.

62. (New) A method for inducing cell death in tumor vascular endothelial cells, comprising administering to an animal having a vascularized tumor at least a first antibody that binds to an aminophospholipid on the luminal surface of tumor vascular endothelial cells in an amount effective to induce cell death in tumor vascular endothelial cells.

63. (New) A method for treating an animal having a vascularized tumor, comprising administering to said animal at least a first antibody that binds to an aminophospholipid on the luminal surface of tumor blood vessels in an amount effective to destroy or occlude at least a portion of said tumor blood vessels upon binding to said aminophospholipid.

Phosphatidylserine is a marker of tumor vasculature and a potential target for anti-cancer drugs[1]

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Abbreviations used in this paper are: TF, tissue factor; VCAM-1, vascular cell adhesion molecule 1; IL-1a, interleukin-1 alpha; DPBS, Dulbecco's phosphate buffered saline; HRP; horseradish peroxidase; AP, alkaline phosphatase; SMC, smooth muscle cells; SCID, severe combined immunodeficient; PS, Phosphatidylserine; CL, cardiolipin; ROS, reactive oxygen species.

ABSTRACT

Phosphatidylserine (PS) is a phospholipid that almost exclusively resides on the inner leaflet of the plasma membrane under normal conditions. PS translocation to the external side of the membrane (PS exposure) is associated with apoptosis, necrosis, cell injury, cell activation and malignant transformation. We previously reported that endothelial cells in tumor vessels of Hodgkin's lymphoma implanted in mice express PS on their external surface. Endothelial cells in normal tissues did not express detectable amounts of PS. In the present study, we analyzed five additional tumor models for the exposure of PS on tumor vasculature and investigated a potential mechanism underlying this phenomenon. Anti-PS antibody specifically localized to tumor blood vessels in all tumors (HT29 human colon adenocarcinoma, NCI-H358 human lung carcinoma, B16 mouse melanoma, 3LL mouse lung carcinoma and Colo 26 mouse colon carcinoma). No localization was detected on normal endothelium. An isotype-matched control antibody directed against a different negatively charged lipid, cardiolipin, did not localize to either tumor or normal endothelium. Frozen tumor sections were examined for the presence of apoptotic cells, using a double labeling technique that detected a pan-endothelial cell marker and the apoptosis markers, active caspase 3 and fragmented DNA (Tunel assay). Neither apoptosis marker was present in tumor endothelium, indicating that PS-positive tumor vessels are seldom apoptotic. Externalization of PS also did not correlate with the maturation status of the vessels, as an abnormal pericytic network was equally evident around PS-positive and PS-negative tumor vessels. Various factors and tumor-associated conditions known to be present in the tumor microenvironment were examined for their ability to cause PS translocation in cultured endothelial cells. Hypoxia/reoxygenation, acidity, thrombin, and inflammatory cytokines all induced PS exposure without causing cytotoxicity. Hydrogen peroxide was also a strong inducer. Possibly, conditions inside tumors generate reactive

oxygen species that induce PS exposure. Combined treatment with inflammatory cytokines and hypoxia/reoxygenation had greater than additive effects, suggesting that factors may interact to give amplified effects on PS-exposure on tumor endothelium *in vivo*. Since PS is absent from the outer surface of normal endothelium, its exposure on tumor vessels could potentially be utilized for tumor vessel targeting and imaging.

INTRODUCTION

Phosphatidylserine (PS) is a phospholipid that almost exclusively resides in the inner leaflet of the plasma membrane under normal conditions (1; 2). PS asymmetry is maintained by an ATP-dependent aminophospholipid translocase that is responsible for inward movement of aminophospholipids (3-5). Loss of PS asymmetry results from the outward movement of PS in the plasma membrane and is caused either by inhibition of the translocase (6; 7) or activation of scramblase, a Ca^{2+} dependent enzyme that transports lipids bidirectionally (8; 9). Loss of PS asymmetry is observed under different pathological and physiological conditions, including programmed cell death (10; 11), cell aging (12), intercellular fusion of myoblasts (13) and trophoblasts (14), cell migration (15; 16), activation of platelets (17-19) and cell degranulation (20). Endothelial cells externalize PS in response to thrombin (21) hyperlipidemia (22), viral infection (23), non-lytic concentrations of complement proteins C5b-9 (24) or exposure to calcium ionophore A23187 and PMA (25). Spontaneous PS exposure has been also observed in malignant cells in the absence of exogenous activators or cell injury (26-28).

Several major consequences follow membrane PS exposure. Phagocytic macrophages recognize, attach and eliminate PS-positive senescent and apoptotic cells (29; 30). PS also mediates attachment of T lymphocytes to thrombin-activated endothelial cells (21). The complement system is activated by PS and contributes to the lysis of PS-positive cells (31). Finally, PS exposure contributes to a procoagulant shift on the endothelium (1; 11) by providing a negatively charged lipid surface for assembly and activation of coagulation complexes (32; 33).

A mouse monoclonal IgM, 3SB, directed against PS has been raised and characterized by Rote and colleagues (17). The antibody binds to PS but not to phosphatidylcholine, phosphatidylethanolamine or cardiolipin (CL). 3SB binds to PS-coated ELISA plates in the presence or absence of serum, indicating that PS binding does not require cofactors. 3SB binds to cells having exposed PS (17). It is possible that it recognizes hexagonally packed PS, which has been reported to be antigenic (34).

We previously discovered that 3SB localized to tumor blood vessels in mice bearing human Hodgkin's disease tumors. This finding indicated that endothelial cells in Hodgkin's tumors, in contrast to those in normal tissues, expressed PS on the external surface of their plasma membrane. In the present study, we determined whether vascular PS exposure is observed in other types of tumors, and investigated the causes of PS translocation. PS exposure on tumor vasculature was present in all of six different tumors growing in mice. PS exposure was not due to apoptosis of tumor endothelium, or to irregularities in their coating with perivascular cells. Studies with cultured endothelial cells showed that hypoxia/reoxygenation, acidity, thrombin, and inflammatory cytokines caused PS exposure without causing cytotoxicity. Hydrogen peroxide did likewise. Hypoxia/reoxygenation, acidity, thrombin and inflammatory cytokines may therefore act individually or collectively in tumors to generate peroxide ions and other reactive oxygen species that induce PS exposure on tumor endothelium.

PS on tumor vessels may provide a target molecule for the vascular targeting or imaging of vessels in solid tumors. Annexin V, an endogenous PS-binding ligand, has been used successfully to image PS-expressing activated platelets in thrombi (35), apoptotic cells in rejecting cardiac allografts, cyclophosphamide-treated lymphomas and anti-Fas antibody-

treated livers in rodents (10). Anti-PS antibodies may be directly cytotoxic to tumor vasculature, or mediate the binding of cytotoxic or coagulation factor to tumor vessels complement, host cells. Also, anti-PS antibodies, annexin V and other ligands that bind specifically to PS on tumor endothelial cells might be used to deliver a cytotoxic drug, radionuclide or coagulant to tumor vessels. Vascular targeting agents directed against markers on mature blood-transporting vessels in tumors have caused destruction of tumor vasculature and major tumor regressions in other systems (36-38). The present studies suggest the use of PS-directed antibodies and immunoconjugates for the vascular targeting or imaging of tumor vessels in man.

MATERIALS AND METHODS

Materials. $Na^{125}I$ was obtained from Amersham (Arlington Heights, IL). Dulbecco's modified Eagle's tissue culture medium and Dulbecco PBS containing Ca^{2+} and Mg^{2+} were obtained from Gibco (Grand Usland, NY). Fetal calf serum was obtained from Hyclone (Logan, Utah). O-phenylenediamine, hydrogen peroxide and thrombin were from Sigma (St. Louis, MO). Flat bottom plates with 24 wells were obtained from Falcon (Becton Dickinson and Co., Lincoln Park, NJ). Recombinant murine interleukin-1 alpha, beta and tumor necrosis factor alpha (TNF α) were purchased from R&D Systems (Minneapolis, MN). Interferon of Universal Type I (hybrid protein that substitutes for all types of interferons) was purchased from PBL Biomedical Laboratories (New Brunswick, NJ). Recombinant hepatocyte growth factor (HGF or scatter factor) and actinomycin D were from Calbiochem. Recombinant VEGF, PDGF-BB, $TGF\beta_1$, interleukin–6 (IL-6),

interleukin-8 (IL-8), interleukin-10 (IL-10) and FGF-1 were purchased from PeproTech (Rocky Hill, NJ).

Antibodies

Mouse monoclonal anti-PS and anti-CL IgM antibodies were raised as described (17). Both antibodies have been extensively characterized with regard to the specificity of their binding to PS and CL respectively (17). MECA 32, a pan mouse endothelial cell antibody, was kindly provided by Dr. E. Butcher (Stanford University, CA) and served as a positive control for immunohistochemical studies. Details of this antibody have been published (39). Rabbit anti-rat immunoglobulin, rat-anti mouse immunoglobulin and goat-anti mouse immunoglobulin secondary antibodies conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP) were purchased either from Daco (Carpinteria, CA) or from Jackson Immunoresearch Labs (West Grove, PA).

Cells

L540Cy Hodgkin lymphoma cells, derived from a patient with end-stage disease, were provided by Prof. V. Diehl (Köln, Germany). HT29 human colon adenocarcinoma and NCI-H358 human non-small cell lung carcinoma were provided by Dr. Adi Gazdar (Southwestern Medical Center, Dallas, TX). B16 mouse melanoma and 3LL mouse lung carcinoma were obtained from American Type Cell Collection (Rockville, MD). Colo 26 mouse colorectal carcinoma was a gift from Dr. Ian Hart (ICRF, London, UK). The mouse brain endothelioma, bEnd.3, was provided by Prof. Werner Risau (Max Plank Institution, Munich, Germany).

METHODS

Tissue Culture

bEnd.3 cells, adult bovine aortic endothelial (ABAE) cells and all tumor cells except L540Cy lymphoma cells were maintained in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 2 units/ml penicillin G and 2 ug/ml streptomycin. L540Cy cells were maintained in RPMI 1640 containing the same additives. Cells were sub-cultured once a week. Trypsinization of bEnd.3 cells was performed using 0.125% trypsin in PBS containing 0.2% EDTA. For *in vitro* studies, endothelial cells were seeded at a density of $1x10^4$ cells/ml in 1 ml of culture medium in 24 well plates and incubated 48-96 hours before being used in the assays. Medium was refreshed 24 hours before each experiment.

Growth of tumor cells *in vivo*

For localization studies, $2x10^7$ L540 human Hodgkin's lymphoma cells or $1x10^7$ cells of other tumor types were injected subcutaneously into the right flank of SCID mice (Charles River, Wilmington, MA). Tumors were allowed to reach a volume of 0.4-0.7 cm^3. A minimum of three animals per tumor group was used. Experiments were performed at least three times.

Detection of PS exposure on tumor endothelium *in vivo*

Detection of exposed PS *in vivo* was performed essentially as previously described (37). Briefly, anti-PS or anti-CL mouse IgM antibodies (30 ug/mouse) were injected intravenously in 200 ul of saline. Thirty minutes later mice were sacrificed and their blood circulation was exsanguinated and perfused with heparinized saline as previously described (40). All major organs and tumor were harvested and snap-frozen for preparation of

cryosections. Mouse IgM was detected using goat anti mouse IgM (μ specific) - HRP conjugate followed by development with carbazole (41). The number of positive vessels per high power field was determined at magnification of x 40. At least 10 random fields per section were examined and the average percentage of positive vessels was calculated.

Detection of apoptosis in tumors *in situ*

The blood circulation of mice was perfused with heparinized saline as previously described (40). Tumors were dissected out and snap-frozen for cryosectioning. Cytosolic and nuclear alterations characteristic for apoptotic cells were detected immunohistochemically by using two markers: active caspase-3 (42) and fragmented DNA (43). Active caspase-3 was detected by a rabbit anti-caspase-3 specific antibody (R&D, Minneapolis, MN) followed by incubation with anti-rabbit IgG conjugated to alkaline phosphatase (AP, Pierce, Rockford, IL). Other tumor sections were analyzed by Tunel assay (ApopTag kit, Oncor, MD) using anti-digoxigenin–alkaline phosphatase conjugate as a detecting reagent. To determine whether tumor endothelial cells express apoptotic markers, sections were sequentially labeled with MECA 32 (anti-endothelial marker) and anti rat-HRP secondary antibody, followed by either anti-caspase-3 antibody or Tunel assay and AP-conjugated secondary reagents. Vessels were visualized by their brown color, using Stable DAB (Research Genetics, Huntsville, AL) as a substrate. Apoptotic cells were identified by their pink-purple color (kit from Research Genetics) created by the phosphatase activity of secondary reagents detecting markers of apoptosis. The pink-purple stain was clearly distinguishable from the brown stain, if both markers coincided. These conditions of double labeling permitted the sequential detection of both enzymes.

Detection of pericytes on tumor frozen sections

To characterize interactions between PS-positive vessels and pericytes, tumor sections were double-labeled by goat anti mouse IgM-HRP (to identify anti-PS localized antibody) followed by anti α-smooth muscle cell actin (α-SMC) antibody (Daco, Carpinteria, CA), a marker of pericytes and vascular smooth muscle cells (SMC)(44; 45). The peroxidase activity was detected by Stable DAB and resulted in brown color. The pericytic marker was detected by anti mouse IgG-AP conjugate and resulted in a pink-purple color. Substrates for both enzymes were from Research Genetics. In other experiments, the endothelium was first detected by MECA 32 IgG and visualized by DAB, followed by anti α-SMC antibody and AP-mediated detection of pericytes/SMC.

Iodination of annexin V

Recombinant human annexin V was purified from E. coli transformed with ET12a-PAP1 plasmid (a gift from Dr. J. Tait, University of Washington, Seattle). The purity of the protein and the binding to PS were confirmed on SDS-PAGE and on PS-coated plastic, respectively. Rabbit polyclonal, affinity-purified anti-annexin V antibody was used to detect annexin V bound to PS. Annexin V was radiolabeled with ^{125}I using Chloramine T as described by Bocci (46). The specific activity was approximately $1x10^6$ cpm per ug of protein, as measured by a Bradford assay (47).

Effect of growth factors, cytokines, inflammatory mediators, hydrogen peroxide, hypoxia and acidic pH on translocation of PS in cultured endothelial cells

Endothelial cells were treated with cytokines or growth factors at the concentrations listed in Table 3. All reagents were diluted in medium containing 10% serum and incubated with the cells at 37°C for 24 hours. To study the effect of hypoxia, cells were seeded on 24 well

plates and were incubated in a humidified normoxic atmosphere (21% O_2, 5% CO_2) for 48 hours before being transferred to a humidified hypoxic atmosphere (1% O_2, 5% CO_2, 94% N_2) in a sealed chamber (Billups Rothenberg inc, Del Mar, Ca). Cells were incubated in a hypoxic chamber for 24 hours at 37°C and the cells were compared to a parallel culture maintained under normoxic conditions. In some experiments, IL-1α (10 ng/ml) and TNFα (20 ng/ml) were added to medium prior to transfer to a hypoxic chamber.

To examine the effect of an acidic microenvironment, cells were exposed to the growth medium lacking bicarbonate, which was adjusted to different pHs (ranged between 7.3 to 6.2) with the required amount of HCl. Cells were incubated at 37°C in the absence of CO_2. Preservation of the medium pH in the presence of cell monolayer during 24 hours period was confirmed in each experiment. Under these experimental conditions all tested reagents were non-toxic to either bovine or mouse endothelial cells and had no effect on cell morphology or viability of the attached monolayer.

Detection of exposed PS on cultured endothelial cells by ^{125}I-labeled annexin V

After treatment with the reagents described above, treated and control cells were incubated with 7.1 pmoles of ^{125}I-labeled annexin V (200 ul/well) in the binding buffer. After 2 hours incubation at room temperature, cells were washed extensively and dissolved in 0.5 M of NaOH. The entire volume of 0.5 ml was transferred to plastic tubes and counted in a gamma counter. Non-specific binding was determined in the presence of 5 mM EDTA and was subtracted from experimental values. The results were expressed as net pmoles of cell-bound annexin V, normalized per $1x10^6$ cells. Maximal binding of annexin V was determined on cells simultaneously treated with actinomycin D and TNFα (50 ng/ml of each component). As has been previously reported, combination of the above agents causes apoptosis and PS exposure in the 100 percent of the treated endothelial cells (48).

Basal binding of ^{125}I-annexin V to untreated cells was determined in the presence of medium with 10% serum. The amount of ^{125}I-annexin V that bound to the untreated cultures was subtracted from that in the treated cultures. The specific increase in the amount of externalized PS was calculated according to the following formula: (net experimental binding / net maximal binding) X 100. Each experiment was performed in duplicate and was performed at least three times.

Detection of exposed PS on endothelial cells *in vitro* by biotinylated annexin V

Endothelial monolayers were washed with DPBS containing Ca^{2+} and Mg^{2+} and fixed with 0.25% of glutaraldehyde diluted in the same buffer. Excess aldehyde groups was quenched by incubation with 50 mM of Na_4Cl for 5 minutes. Cells were washed with DPBS (containing Ca^{2+}, Mg^{2+} and 0.2% gelatin) and incubated with 1 ug/ml of biotinylated annexin V (Pharmingen, San Diego, CA). After 2 hours of incubation, cells were washed with 0.2% gelatin buffer incubated with streptavidin-HRP (1:500 dilution). Detection of PS *in situ* was confirmed by staining with anti-PS IgM. Anti-CL IgM and streptavidin were used as negative controls in these assays. All steps were performed at room temperature. PS-positive cells were detected by addition of carbazole substrate, resulting in insoluble red-brownish precipitate. The number of positive cells per high power field was determined and expressed as a percent of the total number of cells. Six random fields were scored per well and the average was calculated.

RESULTS

PS is a marker of tumor vasculature.

PS exposure on vascular endothelium *in vivo* was detected by a specific anti-PS mouse IgM, (3SB) as previously described (37). A class and species matched antibody, D11 (17),

directed against a different negatively charged phospholipid, CL, served as a negative control. CL exclusively resides in the membrane of mitochondria (49). This anti-CL monoclonal antibody recognizes CL, not PS or other lipids (17).

All six tumors included in this study contained PS-positive vessels (Fig.1 and Table 1). The percentage of PS-positive vessels ranged from 40% in B16 tumors to 10% in Colo 26 tumors. Anti-PS IgM was present on the luminal surface of capillaries and venules in all regions of the tumors. PS-positive vessels appeared to be particularly prevalent in and around regions of necrosis. Positive vessels usually did not show morphological abnormalities that were apparent by light microscopy. Occasional vessels located in necrotic areas showed morphological signs of deterioration. Detection of PS by 3SB was specific since no staining of tumor endothelium was observed with the anti-CL antibody. Anti-PS antibody (but not anti-CL antibody) also localized to necrotic and apoptotic tumor cells. No vascular localization of anti-PS or anti-CL antibodies was observed in normal organs other than the kidneys (Table 1). In the kidneys, tubules were stained in both anti-PS and anti-CL recipients, presumably because of secretion of IgM through this organ. These findings demonstrate that PS is present on the luminal surface of vascular endothelial in various tumors but not in normal tissues.

PS-positive tumor vessels are not apoptotic

A double labeling technique was used to identify apoptotic endothelial cells in tumor sections. Apoptotic cells were identified with two independent markers: an active form of caspase-3, which identifies cytosolic changes in dying cells (42), and fragmented DNA, which identifies cells having nuclear alterations (43). Active caspase-3 was detected by a specific antibody. Fragmented DNA was visualized by Tunel assay (43). Cells positive for apoptotic markers were stained pink by AP-labeled secondary reagents. Endothelial cells in blood vessels were stained brown by HRP-labeled by a pan-endothelial antibody, MECA

32. Both colors were clearly visible on the same cells, if endothelial and apoptotic markers coincided.

Endothelial cells in five out of six types of tumors (HT29, H358, B16, Colo 26, L540) did not display either of the apoptosis markers (Fig. 2, Table 2). The sixth type of tumor, 3LL, displayed a few apoptotic endothelial cells that were located in necrotic areas. In contrast, apoptotic malignant cells were common in all types of tumors. The percentage of apoptotic tumor cells ranged from 1-2% in L540 tumors to 12.6-19.6% in 3LL tumors. There was broad correspondence between the number and location of tumor cells that stained positively for active caspase 3 and for fragmented DNA. However, cells displaying active caspase 3 were about 1.5 times as abundant as those with fragmented DNA, probably because active caspase 3 is an earlier and less specific marker of apoptosis than is fragmented DNA.

Lack of correlation between PS-positive tumor vessels and abnormal distribution of pericytes

We explored whether abnormalities in the perivascular cell coating of tumor vessels might account for their PS exposure by determining whether there was a correlation between the integrity of the coating by pericytes and the positivity for externalized PS. Frozen tumor sections from mice that had been injected with anti-PS antibody or anti-CL antibody were double labeled for the presence of the vascular endothelial cell marker, MECA 32, and a pericytic cell/SMC marker, αSMC-actin. Endothelial cells were stained brown while pericytic cells were stained pink. PS-positive tumor vessels were identified in sequential sections by staining for localized anti-PS IgM (brown) followed by identification of pericytes (pink). In contrast to the continuous pericytic layer surrounding vascular

endothelial cells in normal tissues, vessels in all tumors had a discontinuous and disorganized network of pericytic cells (Fig. 3). Vessels in B16 melanomas and Colo 26 carcinomas almost completely lacked pericytes. HT29 and H358 carcinomas contained pericytes/SMC that were often totally separated from endothelial cells. The L540 lymphoma was the only tumor of those examined that had a major percentage (about 40%) of vessels in which pericytic cells were properly attached.

There was no correlation between irregularities in pericytic cells and exposure of PS on tumor vessels (Fig. 3). PS-positive and PS-negative tumor endothelium had equal proportions of coated vessels, uncoated vessels and vessels with partly detached pericytic layers. Abnormalities in pericyte coating, therefore, do not appear to be the cause of PS translocation on tumor endothelium.

Inducers of PS translocation by endothelial cells *in vitro*

Endothelial cells *in vitro* were treated with non-toxic concentrations of various factors and conditions that are present in the microenvironment of many tumors (50-54). The factors included: angiogenic factors (VEGF, HGF and bFGF) tumor and host cell–derived inflammatory and pro-angiogenic cytokines (1L-1α, IL-1β, TNFα, IL-6, IL-8, IL-10 and interferons), thrombin, hypoxia/reoxygenation , oxygen-reactive species and low pH. These conditions are all known to perturb endothelial cells and could be considered as potential cause of PS translocation. Activation, perturbation and injury of endothelium by these factors and conditions are associated with a rise of intracellular Ca^{2+} concentration. The rise in intracellular Ca^{2+} might activate scramblase (9) and simultaneously inhibit aminophospholipid translocase (6), leading to accumulation of PS on the external side of the membrane.

Mouse bEnd.3 or bovine ABAE cells were treated for 24 hours with different concentrations of potential inducers of PS translocation. PS externalization was quantified by measuring ^{125}I-annexin V binding. The amount of annexin V binding was compared with that of cells in which apoptosis of 100% of cells had been induced by combined treatment with actinomycin D and TNF-α. Actinomycin D and TNF-α induced the binding of 6.2 pmoles of annexin V per 10^6 cells (3.8 x 10^6 molecules of annexin V per cell) on both cell types, in good agreement with literature reports (55). This value was taken as the maximal amount of externalized PS.

Untreated cells were largely devoid of externalized PS, as judged by annexin V or anti-PS antibody binding (Table 3, and Fig. 4A). The basal binding in the presence of growth medium alone was 0.44 and 0.68 pmoles of ^{125}I-annexin V for ABAE and bEnd.3 cells, respectively. This corresponds to 7.06% and 10.9% of the maximal binding for ABAE and bEnd.3 cells, respectively, which correlated well with the finding that approximately 10% of cells bound biotinylated annexin V under the same conditions. VEGF, HGF, FGF, TGFβ$_1$, PDGF, IL-6, IL-8 and IL-10 did not increase binding of ^{125}I-annexin V above the basal level for untreated cells. Inflammatory mediators (IL-1α, IL-1β, TNFα and interferon) caused a small but reproducible increase in PS translocation that ranged from 6 to 8% of the maximal level for ABAE cells and from 7 to 14% for bEnd3 cells (Table 3). Hypoxia/reoxygenation, thrombin or acidic external conditions (pH 6.8-6.6) induced a moderately high externalization of PS that ranged from 8 to 20% of the maximal level for ABAE cells and from 17 to 22% of the maximal level for bend.3 cells. The largest increase in PS translocation was observed after treatment with 100 to 200 uM of hydrogen peroxide. This treatment caused nearly complete (95%) externalization of PS in both cell types as judged by ^{125} I-annexin V binding (Table 3). More than 70% of ABAE and bEnd.3

cells bound biotinylated annexin V, as judged immunohistochemically (Fig. 4B). PS-expressing endothelial cells generated by treatment with hypoxia/reoxygenation, thrombin, acidity, TNFα, IL-1 or H_2O_2 remained attached to the matrix during time period of the assay (24 hours), retained cell-cell contact (Fig. 4B) and retained their ability to exclude trypan blue dye. Normal PS orientation was restored 24 to 48 hours later in the majority of the cells. These results indicate that mild oxidative stress, created by direct application of H_2O_2 or indirectly by hypoxia/reoxygenation, acidity, thrombin, or inflammatory cytokines, triggers a transient translocation of PS on viable endothelial cells.

Combined Effects of Inflammatory Cytokines and Hypoxia/Reoxygenation on PS-Exposure by Endothelial Cells *In Vitro*

Enhanced PS exposure was observed when ABAE cells were subjected to hypoxia/reoxygenation in the presence of IL-1α or TNFα. In the absence of the cytokines, hypoxia/reoxygenation increased PS-exposure by ABAE cells to 15% of the maximum level for cells treated with apoptotic concentrations of actinomycin D and TNFα. In the presence of subtoxic concentrations of IL-1α or TNFα, hypoxia/reoxygenation increased PS-exposure to 26% and 33% respectively of the maximum (Fig. 5). Cytokines in the absence of hypoxia/reoxygenation increased PS-exposure by less than 7% indicating that the combination of cytokines and hypoxia/reoxygenation had greater than additive effects on PS-exposure. Thus, in tumors, the PS-inducing effect of hypoxia/reoxygenation may be amplified by inflammatory cytokines and possibly by such other stimuli as acidity and thrombin.

DISCUSSION

The major finding to emerge from this study is that vascular endothelial cells in tumors externalize PS to their luminal surface where it can be bound by a specific anti-PS antibody *in vivo*. PS is absent from the external surface of vascular endothelial cells in normal tissues, suggesting that PS-recognizing antibodies, annexin V and other ligands might be used for delivering cytotoxic drugs, coagulants or radionuclides for the selective destruction or imaging of vessels in solid tumors.

PS-positive tumor endothelium appears to be viable. It does not display markers of apoptosis, it is morphologically intact and metabolically active, as indicated by its expression of VCAM-1, E-selectin and other rapidly turned-over proteins. Although often regarded as an indicator of apoptosis, PS exposure has been observed in several types of viable cells, including malignant cells (27; 28; 55), activated platelets (17), and embryonic trophoblasts at various stages of migration, matrix invasion and fusion (14). Recent studies suggest that constitutive PS exposure on malignant cells may occur because expression of truncated (less active) aminophospholipid translocase, as detected by epitope-specific antibodies against the putative PS translocase, ATPaseII[4]. Lack of correlation between PS exposure and commitment to cell death has been also shown on pre-apoptotic B lymphoma cells that restored PS asymmetry and grew normally after removal of pro-apoptotic stimulus (56). In normal viable cells, PS exposure is probably triggered by surface events, such as ligand-receptor interactions, that induce Ca^{2+} fluxes into the cells (57). Ca^{2+} fluxes activate scramblase (9) and simultaneously inhibit aminophospholipid translocase (6).

To shed light on the mechanism of PS exposure on tumor endothelial cells, a series of experiments was performed in which endothelial cells *in vitro* were treated with various factors and conditions known to be present in the tumor microenvironment. The objective was to identify factors that induce PS exposure on endothelial cells without causing cytotoxicity in order to mimic the situation in tumors *in vivo*. Hypoxia followed by reoxygenation, acidity, and thrombin increased PS exposure on viable endothelial cells to between 10 and 22% of the level seen when all cells are apoptotic. Inflammatory cytokines (TNFα and IL-1α) also caused a weak but definite induction of PS exposure. The possibility that these conditions are in fact the major inducing stimuli in tumors *in vivo* is suggested by the following: i) PS positive endothelium is prevalent in and around regions of necrosis where hypoxia, acidity, thrombosed blood vessels, and infiltrating host leukocytes are commonly observed; ii) the finding that hypoxia/reoxygenation amplifies the weak PS-exposing activity of TNFα and IL-1 on endothelial cells *in vitro* (Fig. 5) correlates with the situation *in vivo* in tumors where hypoxia and cytokine-secreting tumor and host cells co-exist; iii) hypoxia/reoxygenation and thrombin have been reported to generate reactive oxygen species (e.g. peroxides) in endothelial cells through activation of NADPH oxidase-like membrane enzyme (58; 59). Hydrogen peroxide was the most powerful inducer of PS exposure on cultured endothelial cells found in the present study, providing indirect support for the involvement of reactive oxygen species. Based on these findings we propose the following cascade of events leading to PS exposure on tumor vessels, which indirectly contributes to tumor-associated thrombosis (Fig. 6). Hypoxia/reoxygenation in combination with inflammatory cytokines, thrombin and acidity are responsible in part for generation of ROS by endothelial and tumor cells. ROS are also produced by infiltrating host cells (macrophages, neutrophils and granulocytes) that are

attracted by necrosis and tumor-derived cytokines. Tumor endothelial cells respond to rising ROS concentration by transferring PS to the external side of the membrane. The externalized PS provides the negative phospholipid surface upon which coagulation factors concentrate and assemble. This confers the procoagulant status to the tumor endothelium that has long been recognized. PS also provides an attachment site for circulating macrophages (29), T lymphocytes (21) and polymorphonuclear cells that assist in leukocyte infiltration into tumors. Adherence of activated macrophages, polymorphonuclear cells and platelets to PS on tumor endothelium may lead to further secretion of reactive oxygen species and further amplification of PS exposure.

Anti-PS antibodies might be used for cancer therapy in several ways. Unconjugated antibodies might directly suppress tumor endothelial cell growth or survival by interfering with critical PS-dependent surface functions. This hypothesis is supported by the studies on viable B cells demonstrating that PS neutralization inhibits signaling from the cell surface via B cell receptor (57). Anti-PS antibodies might also mediate toxicity by binding complement and cytotoxic cells, by inducing apoptosis or by promoting pro-thrombotic status of tumor vascular endothelium. In certain autoimmune disorders, anti-aminophospholipid antibodies cause normal tissue damage by analogous mechanisms (60-62). Also, anti-PS antibodies or ligands might be linked to various effector molecules (e.g. cytotoxic drugs, radionuclides, coagulants) to create vascular targeting agents that destroy or occlude blood vessels in solid tumors. Such agents have been shown to be highly effective, and sometimes curative, in mice with large solid tumors (36; 38).

PS on tumor vessels is attractive as a target for several reasons: it is abundant (minimum of 3×10^6 molecules per cell); it is on the luminal surface of tumor endothelium, which is

directly accessible for binding by vascular targeting agents in the blood; it is present on a high percentage of tumor endothelial cells in diverse solid tumors, and it is absent from endothelium in all normal tissues examined to date. Unconjugated antibodies, vascular targeting agents and imaging agents directed against PS on tumor vasculature potentially could have utility for cancer treatment in man.

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Table 1 Specific localization of anti-PS and anti-CL antibodies to tumor vessels mice

Tissue	Anti-PS[a]	Anti-cardiolipin
L540 tumor	++[b]	-
H358 tumor	++	-
HT29 tumor	+	-
B16 tumor	+++	-
3LL tumor	++	-
Colo 26 tumor	++	-
Adrenal	-	-
Brain	-	-
Heart	-	-
Kidney	-[c]	-[c]
Intestine	-	-
Liver	-	-
Lung	-	-
Pancreas	-	-
Spleen	-	-
Testis	-	-

[a] Localization of anti-PS or anti-CL antibody in tumor bearing mice was determined by injecting the antibody, perfusing the blood circulation of the mice with saline and detecting the antibody on sections of the tissues by using an anti-mouse IgM - peroxidase conjugate.

[b] Intensity of staining was compared to pan-endothelial marker MECA 32; - indicates no staining; + weak (fewer than 5% positive vessels) ; ++ moderate (5 to 20 %); +++ (20 to 40 %) strong.

[c] Non-antigen specific tubular staining was visible in both anti-PS and anti-CL recipients.

Table 2 **Expression of apoptotic markers in tumors**

Tumor type	Active caspase-3		Tunel assay	
	Tumor cells (% of total) [a]	Tumor vessels	Tumor cells (% of total)	Tumor vessels
3LL	19.8	<1.0 [b]	12.6	0
HT29	13.7	0	7.8	0
H358	5.8	0	4.3	0
Colo 26	5.3	0	4.1	0
B16	4.2	0	3.5	0
L540	2.3	0	1.6	0

[a] The percentage of tumor cells or tumor blood vessels that were positive for either caspase-3 or Tunel was determined in ten high power fields per section. The fields were randomly selected along two perpendicular directions from the edges through the center of the tumor. The average of the percentage of positive cells or vessels in tumor from 3 mice is presented.

[b] Occasional vessels (1 of >100) in the necrotic area of 3LL tumor displayed both markers of apoptosis.

Table 3 Effect of cytokines, growth factors and stress conditions on exposure of PS on endothelial cells *in vitro.*

Treatment	Concentration[a]	^{125}I-Annexin V (% of Max binding)[b]	
		ABAE cells	bEnd.3 cells
Medium with 10% serum	N/A[c]	0	0
Actinomycin D + TNF α	50 ng/ml each	100	100
VEGF	20 ng/ml	0	0
βFGF	20 ng/ml	0	0
Scatter factor	40 ng/ml	0	0
TGF β_1	20 ng/ml	0	0
PDGF-BB	20 ng/ml	0	0
IL-10	20 ng/ml	0	0
IL-8	20 ng/ml	0	0
IL-6	20 ng/ml	0	0
IL-1α	10 ng/ml	6.4	7.5
IL-1β	10 ng/ml	5.8	5.5
Interferon	40 ng/ml	8.6	2.8
TNFα	20 ng/ml	7.4	13.7
Thrombin	50 nM	8.8	17.4
Hypoxia	1% O_2	15.0	22.5
pH 6.6[d]	N/A	20.2	18.9
Hydrogen peroxide	100 uM[e]	95.5	98.4

[a] Concentrations of cytokines, growth factors and thrombin were selected from literature values to have maximal stimulatory effect on cultured endothelial cells. These concentrations did not cause toxicity over the period of the test (24 hours) as judged by morphological appearance, a lack of detachment, and a lack of uptake of trypan blue.

[b] Binding of ^{125}I-annexin V was performed as described under Methods. Basal binding was determined in the presence of growth medium alone. Maximal PS exposure was determined after induction of apoptosis by the combined treatment with actinomycin D and TNF α. Untreated ABAE and bEnd.3 cells bound 0.44 and 0.68 pmoles of ^{125}I-Annexin V, respectively. Maximal binding was 6.2 pmoles of ^{125}I-annexin V for both cell types (equivalent to 3.8×10^6 molecules per cell) The percentage of increase of annexin V binding was calculated according to the following formula: (net experimental binding / net maximal binding) X 100. Average of duplicates from three separate experiments is presented. SE was less than 5%.

[c] Not applicable.

[d] Cells were exposed to the growth medium lacking bicarbonate that had been adjusted to pH 6.6 with 1N HCl. Cells were incubated at 37^0C in the absence of CO_2.

[e] The maximal concentration of H_2O_2 that did not cause cytotoxicity under chosen conditions.

Figure Legends:

Fig. 1. Localization of anti-PS antibody to vascular endothelial cells in L540 human Hodgkin's lymphoma, 3LL murine lung carcinoma and B16 murine melanoma tumors in mice. Tumor-bearing SCID mice were injected intravenously with 20 ug of anti-PS or anti-CL mouse IgM. The blood circulation was perfused with saline one hour later. Mice were sacrificed one hour later and tumor and organs were harvested and snap-frozen. Mouse IgM was detected on frozen sections using goat anti-mouse IgM-peroxidase conjugate. Anti-PS antibody specifically localized to blood vessels (indicated by arrows) in all tumors. No localization was observed in mice injected with control anti-CL IgM.

Fig. 2. Lack of apoptotic vascular endothelial cells in 3LL and L540 tumors. Frozen sections of 3LL and L540 tumors were double labeled with a pan-endothelial cell antibody, MECA 32 to visualize blood vessels, and with anti-apoptotic markers detecting either active caspase-3 or fragmented DNA (Tunel assay). MECA 32 staining of vessels is indicated by the brown color (arrows). Expression of apoptotic markers is indicated by the pink color (arrowheads). Both apoptotic markers are present in some tumor cells but are absent from tumor endothelium.

Fig 3. Pericyte-endothelial cell interactions in PS-positive and PS-negative vessels in 3LL tumors. Mice bearing 3LL tumors were injected with 20 ug of anti-PS IgM. PS-positive vessels (*upper panel*) were detected as described above. PS-negative vessels (defined as MECA 32-positive, anti-PS-negative) were identified on serial sections of the same tumor (*lower panel*). Vessels were labeled brown with the pan-endothelial cell marker. Pericytes were identified with anti-αSMC actin antibody and were stained pink (arrowheads). Representative fields of PS-positive, pericyte-negative (*upper left*), PS-positive, pericyte-positive (*upper right*), PS-negative, pericyte-negative (*lower left*) and PS-negative, pericyte-positive (*lower right*) vessels are shown. Note the detachment of the pericytic layer from tumor endothelium and the uneven distribution of pericytes along tumor vessels (*upper and lower right*).

Fig. 4. Externalized PS on bEnd.3 mouse endothelial cells treated with 100 uM of hydrogen peroxide. A) untreated bEnd.3 cells; B) bEnd.3 cells after treatment with 100 uM of hydrogen peroxide. PS is absent from the surface of untreated cells but becomes externalized after treating the cells with H_2O_2. PS-positive cells remain attached to the substratum, retain cell-cell contact and other morphological signs of viability.

Fig. 5. Synergistic effect of hypoxia and inflammatory cytokines on PS exposure. bEnd.3 cells were treated for 24 hours with IL-1 α and TNF α under normoxic and hypoxic conditions. PS externalization was determined on viable endothelial monolayer by measuring binding of ^{125}I-annexin V. The increase in PS exposure was calculated as explained under "Materials and Methods".

Fig. 6. Hypothesis for induction of PS exposure on tumor vessels and its contribution to the procoagulant shift of the tumor endothelium.

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Philip E. Thorpe and Sophia Ran

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SUBMISSION OF FORMAL DRAWINGS

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Assistant Commissioner for Patents
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Sir:

Applicants hereby submit the formal drawings for the above-referenced application (Figures 1A through 4B on 3 sheets) and request that these drawings be accepted for filing.

Respectfully submitted,

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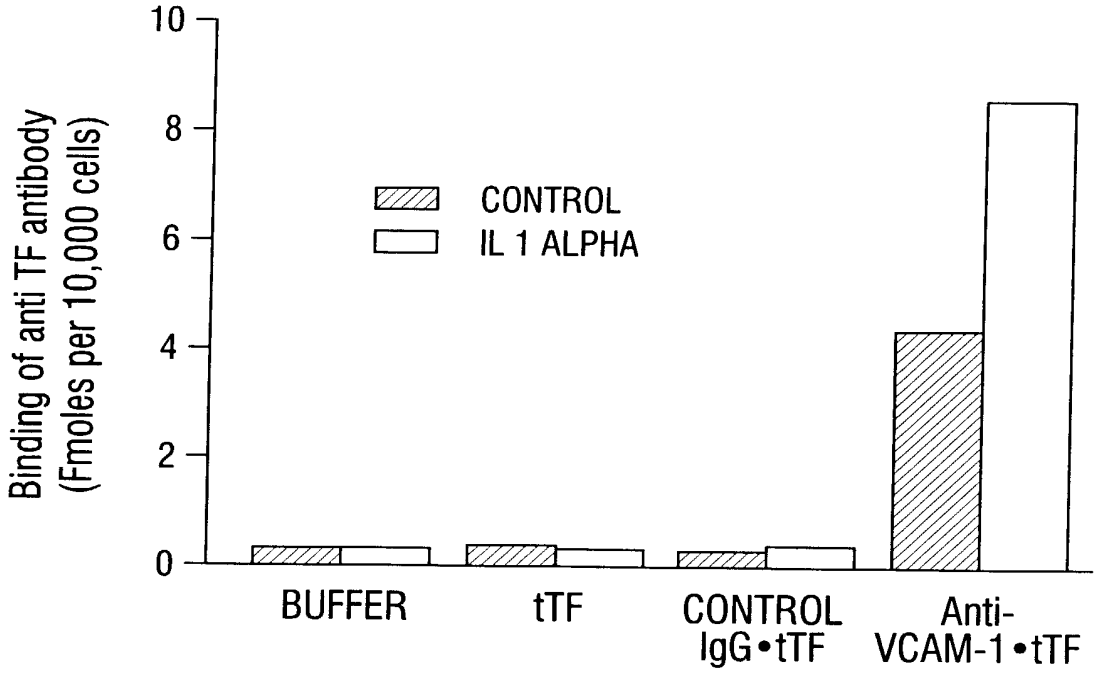


FIG. 1A

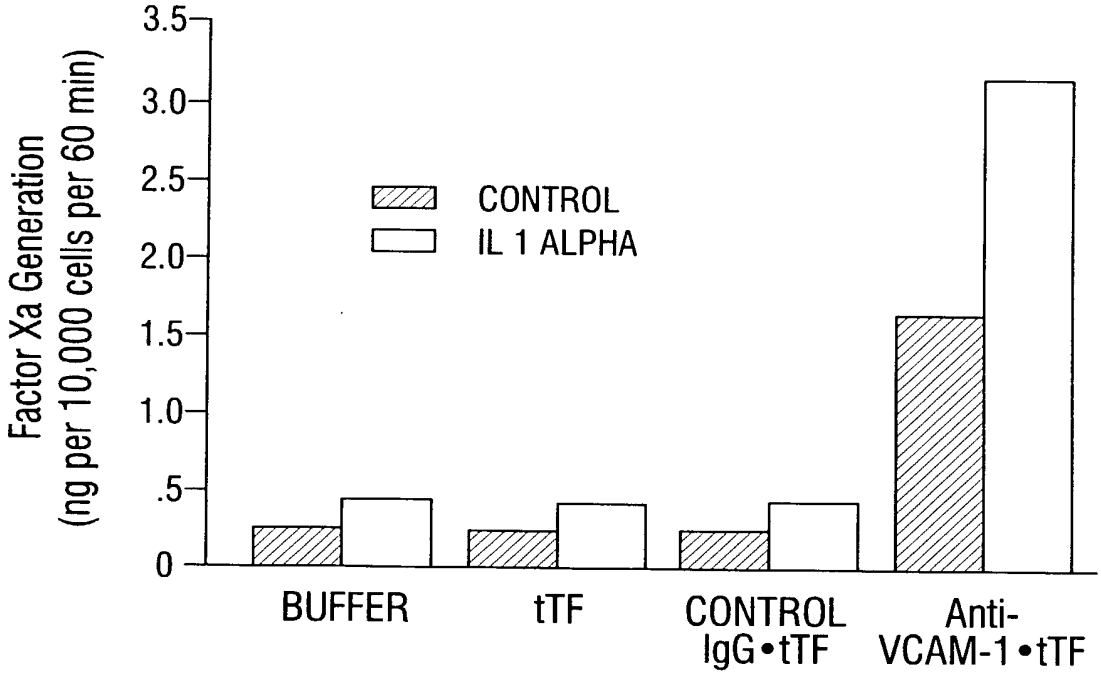
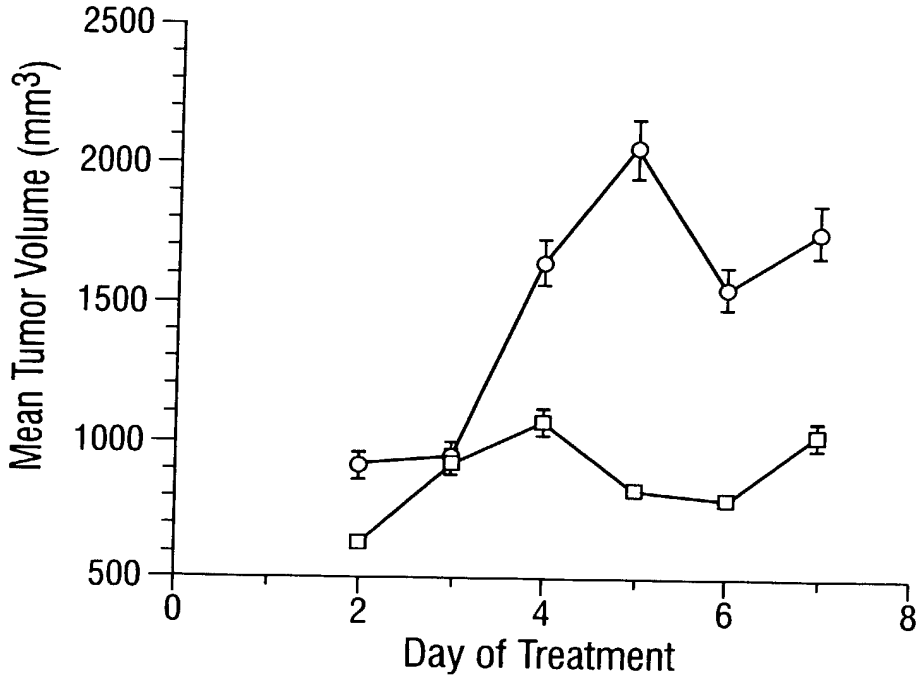


FIG. 1B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2000
1500
1000
500
Mean Tumor Volume (mm3)
0
2
4
6
8
Day of Treatment

FIG. 4A

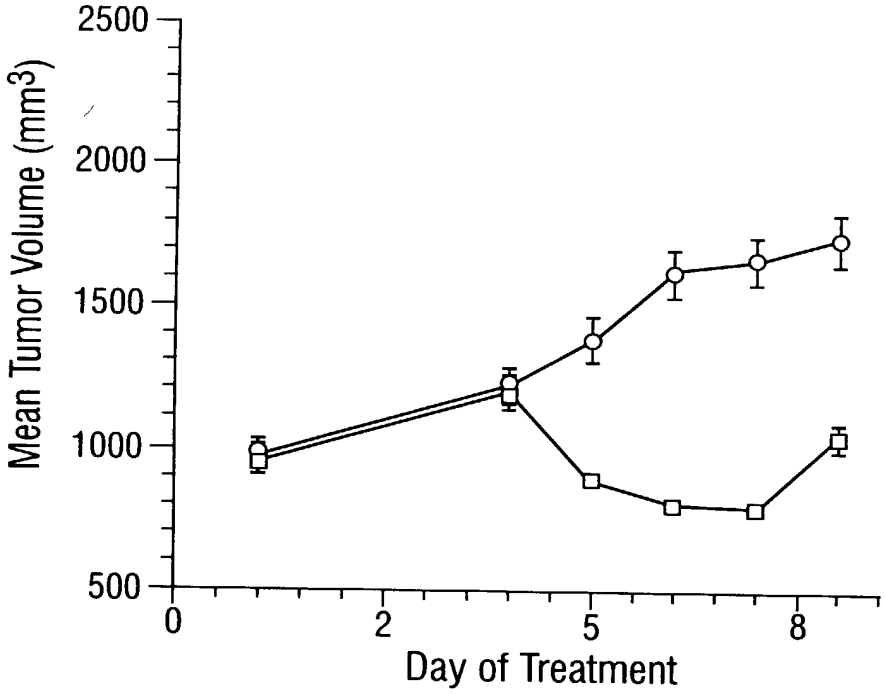
2500
2000
1500
1000
500
Mean Tumor Volume (mm3)
0
2
5
8
Day of Treatment

FIG. 4B